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Zebrafish as a model to assess the teratogenic potential of nitrite

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Abstract:	High nitrate levels in the environment can result in congenital defects or miscarriages in humans. Presumably, this is due to the conversion of nitrate to nitrite by gut and salivary bacteria. However, in other mammalian studies, high nitrite levels do not cause birth defects, although they can lead to poor reproductive outcomes. Thus, the teratogenic potential of nitrite is not clear. It would be useful to have a vertebrate model system to easily assess teratogenic effects of nitrite or any other chemical of interest. Here, we demonstrate the utility of zebrafish (Danio rerio) to screen molecules for toxicity and embryonic defects. Zebrafish embryos are fertilized externally and have rapid development, making them a good model for teratogenic studies. We show that increasing the time of exposure to nitrite negatively affects survival. Increasing the concentration of nitrite also adversely affects survival, whereas nitrate does not. For embryos that survive nitrite exposure, various defects can occur, including pericardial edema, swim bladder noninflation, and craniofacial malformation. Our results indicate that the zebrafish is a convenient system for studying the teratogenic potential of nitrite. This approach can easily be adapted to test other chemicals for their effects on early vertebrate development.
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TITLE
Zebrafish as a model to assess the teratogenic potential of nitrite

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SHORT ABSTRACT
Exposure to teratogens can cause birth defects. Zebrafish are useful for determining the teratogenic potential of chemicals. We demonstrate the utility of zebrafish by exposing embryos to various levels of nitrite and also at different times of exposure. We show that

nitrite can be toxic and cause severe developmental defects.

LONG ABSTRACT
High nitrate levels in the environment can result in congenital defects or miscarriages in humans. Presumably, this is due to the conversion of nitrate to nitrite by gut and salivary bacteria. However, in other mammalian studies, high nitrite levels do not cause birth defects, although they can lead to poor reproductive outcomes. Thus, the teratogenic potential of nitrite is not clear. It would be useful to have a vertebrate model system to easily assess teratogenic effects of nitrite or any other chemical of interest. Here, we demonstrate the utility of zebrafish (*Danio rerio*) to screen molecules for toxicity and embryonic defects. Zebrafish embryos are fertilized externally and have rapid development, making them a good model for teratogenic studies. We show that increasing the time of exposure to nitrite negatively affects survival. Increasing the concentration of nitrite also adversely affects survival, whereas nitrate does not. For embryos that survive nitrite exposure, various defects can occur, including pericardial edema, swim bladder noninflation, and craniofacial malformation. Our results indicate that the zebrafish is a convenient system for studying the teratogenic potential of nitrite. This approach can easily be adapted to test other chemicals for their effects on early vertebrate development.

INTRODUCTION
Teratogenesis is a process that disrupts the normal development of an embryo or fetus by causing permanent structural and functional abnormalities, growth retardation, or miscarriage in severe cases[1]. It can be caused by certain natural agents (teratogens), which interfere with embryonic development in multiple ways[2]. During human fetal development, common teratogens such as radiation, infectious agents, toxic metals, and organic chemicals have been reported to cause defects in epicanthic folds (the skin fold in the upper eye lid) and clinodactyly (curved finger or toe) through morphogenetic errors[1].

Understating the molecular mechanism of teratogenesis is the first step towards developing treatment and prevention. Several vertebrate models such as the African clawed frog (*Xenopus laevis*) and zebrafish (*Danio rerio*) have been used to determine the molecular pathways affected by teratogens. Previous studies have used zebrafish as a model for epidemiology, toxicology and teratogenesis[3-7]. Scholz *et al*. considered zebrafish as a “gold standard” for environmental toxicity assessment. This is due, in part, to the transparency of the zebrafish embryo, which allows researchers to visualize the developmental defect as it occurs[8]. Approximately 70% of human genes have orthologues in zebrafish, making zebrafish a desirable vertebrate model for studying human defects[9].

Some epidemiological studies have suggested that nitrate and nitrite, commonly present in farm foods and water, are associated with birth defects or spontaneous abortions[10,11], while other studies do not support this association[12]. Nitrate (NO_3^-) and nitrite (NO_2^-) are naturally present in soil and water. They are a source of nitrogen for plants and are a part of the nitrogen cycle[13]. Foods such as green beans, carrots, squash, spinach, and

beets from farms that use fertilizers high in nitrate have significantly augmented levels of nitrate and nitrite[7]. Milk from cows fed with high nitrate foods and fish in high nitrate water (mainly from soil runoff[30]) can lead to humans consuming large amounts of nitrate and nitrite[14]. Nitrate and nitrite are also commonly used in food preservation, which dramatically increases the amount ingested by humans[12]

Optimal levels of nitrate and nitrite play fundamental roles in physiological processes like vascular homeostasis and function, neurotransmission and immunological host defense mechanisms[13-15]. However, exposure to high levels of nitrate and nitrite may lead to adverse effects, especially in infants and children[16]. Ingested nitrate is further converted to nitrite in the oral cavity by microflora and in the gastrointestinal tract by intestinal microflora[17].

Nitrate puts infants at a high risk for blue baby syndrome by oxidation of hemoglobin to methemoglobin, impairing hemoglobin from its oxygen carrying ability[18]. This results in the blue color of skin that extends to peripheral tissues in more severe cases. Inhibited oxygenation of tissues results in other symptoms, most severely leading to coma and death[19,20]. Similar symptoms are observed in babies and adults at higher concentrations of nitrate[21]. Elevated levels of methemoglobin in adults due to nitrite poisoning results in cyanosis, headache, breathing disorders[31], and if not treated death due to complications related to vital tissue hypoxia[32,33].

Nitrate ingested at higher levels can also result in various health complications. Childhood diabetes, recurrent diarrhea, and recurrent respiratory tract infections in children have been linked with high nitrate intake[11,17,22]. Chronic exposure to a high level of nitrate is associated with urination and spleen hemorrhaging. Acute high dose exposure to nitrates can lead to a wide spectrum of medical conditions like abdominal pain, muscle weakness, blood in stools and urine, fainting, and death[11]. Prenatal exposure to nitrate at high levels has been associated with neural tube and musculoskeletal defect[11].

A recent report showed that treating zebrafish embryos with nitrite led to yolk sac edema, craniofacial and axial malformations, and swim bladder noninflation[5]. In this study, we demonstrate a method for treating zebrafish embryos with nitrate and nitrite to determine their teratogenic potential. Embryos were exposed to nitrite at different concentrations and different lengths of time. Ethanol was used as a positive control, since it is an established teratogen[23]. Our method showed that both high concentrations and long exposure times to nitrite were detrimental to survival and resulted in various phenotypes, ranging from mild (edema) to severe (gross developmental defects). Therefore, the zebrafish is a useful model for directly exploring the potential teratogenic effects of nitrate and nitrite on embryos to complement epidemiological studies.

PROTOCOL

The procedures described in this protocol were approved by the Institutional Animal Care and Use Committee at the Indiana University of Pennsylvania.

1. Harvest embryos

1.1) Maintain zebrafish at 28.5 °C, pH 7, conductivity between 500-1500 µS, and a light/dark cycle of 14 hours light and 10 hours dark[24]. Use wild-type strains such as Tü, AB or Tü/AB hybrid. Different strains may respond differently to chemical treatment[25].

1.2) Set up the fish for mating the night before harvesting eggs by adding fish system water into a mating tank. Add a male and female fish into the tank and separate the two fish with a divider. Each fish pair will produce a range of 50-300 eggs. To ensure that enough eggs will be produced, set up 30 pairs of fish. Typically, about 50% of fish pairs at the prime mating age (6-9 months old) will produce eggs, resulting up to 200 eggs per pair and a maximum of up to 3,000 eggs for this experiment.

1.3) The next morning after the light turns on, remove the divider to initiate mating. Check the mating tanks for eggs every 15 minutes.

1.4) Once the fish lay eggs, harvest all embryos using a tea strainer and combine them into one large container with E3 buffer (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$). At 1.5 hpf, remove and discard unfertilized eggs with a plastic transfer pipet under a dissecting microscope. Unfertilized eggs are opaque while fertilized eggs are transparent. Unfertilized eggs are opaque while fertilized eggs are transparent[34].

1.5) Transfer 50 embryos into a 100 x 15 mm glass Petri dish containing 50 mL E3 buffer for each treatment condition. A total of 33 dishes are needed for 11 treatment conditions and 3 replicates.

2. Treating embryos

2.1) Carry out treatments at 2 hours post-fertilization (hpf)[35]. Incubate embryos/larvae at 28.5 °C and examine the larvae at 120 hpf. Perform three replicates of each treatment condition for statistical analysis.

2.2) For 3 Petri dishes (each containing 50 embryos) at 2 hpf, remove the E3 buffer with a transfer pipet and add 50 mL of 300 mM ethanol diluted in E3 buffer. Cover the Petri dishes with Parafilm to minimize vitalization of ethanol.

2.2.1) Continue exposing the embryos to ethanol for 22 hours. Then remove the ethanol with a transfer pipet. Add 50 mL of E3 buffer and swirl the dish several times to wash out the ethanol. Remove this E3 buffer with a transfer pipet and repeat the washing step 2 more times.

2.3) For 3 Petri dishes (each containing 50 embryos) at 2 hpf, remove the E3 buffer with a transfer pipet and add 50 mL of new E3 buffer.

2.4) For 9 Petri dishes (each containing 50 embryos) at 2 hpf, remove the E3 buffer with

a transfer pipet and add 50 mL of 1,000 mg/L sodium nitrite dissolved in E3 buffer. Confirm the concentration of the stock nitrite solution beforehand using a modification of the diazotization (USEPA Method 354.1) spectrophotometric method[28].

2.4.1) Continue exposing 3 dishes for 46 hours, 3 dishes for 70 hours and 3 dishes for 94 hours. Replace the nitrite solution with freshly made nitrite solution daily.

2.4.2) After each exposure time, remove the nitrite with a transfer pipet. Add 50 mL of E3 buffer and swirl the dish several times to wash out the nitrite. Remove this E3 buffer with a transfer pipet and repeat the washing step 2 more times.

2.5) For 3 Petri dishes (each containing 50 embryos) at 2 hpf, remove the E3 buffer with a transfer pipet and add 50 mL of 200 mg/L sodium nitrite. Repeat this with 400, 600, 800, and 1,000 mg/L of sodium nitrite.

2.5.1) Continue exposing the embryos to nitrite for 70 hours. Replace the nitrite solution with freshly made nitrite solution daily.

2.5.2) After the exposure time, remove the nitrite with a transfer pipet. Add 50 mL of E3 buffer and swirl the dish several times to wash out the nitrite. Remove this E3 buffer with a transfer pipet and repeat the washing step 2 more times.

2.6) For 3 Petri dishes (each containing 50 embryos) at 2 hpf, remove the E3 buffer with a transfer pipet and add 50 mL of 1,000 mg/L sodium nitrate dissolved in E3 buffer. Confirm the concentration of the stock nitrate solution beforehand using a modification of the cadmium reduction (USEPA Method 353.3) spectrophotometric method[28].

2.6.1) Continue exposing the embryos to nitrate for 70 hours. Replace the nitrate solution with freshly made nitrate solution daily.

2.6.2) After the exposure time, remove the nitrate with a transfer pipet. Add 50 mL of E3 buffer and swirl the dish several times to wash out the nitrate. Remove this E3 buffer with a transfer pipet and repeat the washing step 2 more times.

2.7) During each day of the exposure, count the number of dead embryo/larvae using a stereomicroscope. Signs of death include lack of heartbeat and blood circulation, or lack of motility after 1 minute of observation. Remove dead embryos/larvae with a transfer pipet to reduce contamination of the E3 buffer.

2.8) When experiments end at 120 hpf, euthanize the larvae.

2.8.1) Remove the E3 buffer with a transfer pipet. Then add 50 mL of 0.2% MS-222 (buffered to pH 7) and wait for 10 minutes.

2.8.2) Remove the MS-222 with a transfer pipet. Add 50 mL of E3 buffer and swirl to wash out the MS-222.

2.8.3) Remove the E3 buffer with a transfer pipet and add 20 mL of 4% paraformaldehyde (PFA) to fix the larvae. Swirl the dish several times. Use a transfer pipet to transfer the larvae into a glass vial along with enough PFA to fill the vial. Store the vials in the refrigerator overnight.

2.9) Take pictures of fixed larvae using a stereoscope with a digital camera. Use 30X magnification, ISO 200, and 200 ms exposure time. Orient the larvae so that the anterior to the left and the dorsal is to the top of the field.

REPRESENTATIVE RESULTS

Exposure to 300 mM ethanol for 22 hours had no effect on survival (data not shown), consistent with previous reports[5,23,26]. This is expected, as ethanol is a known teratogen and served as a positive control. Observed phenotypes included pericardial edema, swim bladder noninflation (Figure 1), craniofacial defects, and developmental delay (data not shown).

Treatment with nitrite resulted in mild to severe effects on survival, depending on the time of exposure. For example, exposure to 1,000 mg/L for 94 hours severely affected survival compared to shorter exposure times (Figure 2).

We also assessed the effect of different nitrite concentrations on survival. Embryos were exposed for 70 hours to 200, 400, 600, 800, and 1,000 mg/L. Survival rates were lower when exposed to high concentrations of nitrite, whereas nitrate did not have an effect on survival (Figure 3). Phenotypes for nitrite-treated larvae resembled that of ethanol treated embryos (Figure 4).

Figure 1. Developmental effects of ethanol treatment.
Embryos treated with ethanol showed pericardial edema (arrow), swim bladder noninflation (dashed line), yolk sac edema (arrowhead), and craniofacial defects (data not shown). Images were taken at 96 hpf.

Figure 2. Survival of 1,000 mg/L nitrite treatment after different times of exposure.
Embryos were exposed to 1,000 mg/L nitrite at 2 hpf. Nitrite was washed out after 46, 70, and 94 hours of exposure, and survival rate was calculated. Increased exposure time resulted in decreased survival rate. Standard deviations: Untreated = 24; 46 hr = 6; 70 hr = 6; 94 hr = 0.9.

Figure 3. Survival after exposure to different nitrite concentrations.
Embryos were exposed for 70 hours to increasing concentrations of nitrite. Higher concentrations of nitrite resulted in lower survival rates. Nitrate had no effect even at the highest concentration of 1,000 mg/L after exposure for 70 hours. Standard deviations for nitrite: Untreated = 19; 200 mg/L = 16; 400 mg/L = 21; 600 mg/L = 20; 800 mg/L = 14; 1,000 mg/L = 12. Standard deviation for nitrate equals 4.

Figure 4. Developmental effects of nitrate and nitrite.

Embryos treated with 1,000 mg/L nitrate (middle panel) had no effect compared to the untreated control (top panel). Nitrite treatment at 1,000 mg/L resulted in gross developmental defects in addition to similar phenotypes observed in the ethanol treatment (bottom panel). Images were taken at 120 hpf.

DISCUSSION

The method described here demonstrates the utility of zebrafish in assessing the teratogenic potential of nitrite and nitrate. Compared to other vertebrates, zebrafish have advantages that include high fecundity, external fertilization, optical transparency, and rapid development. Available mutants that lack pigmentation (such as the casper zebrafish[36]) also help to enhance visibility of internal organs. It is also easy to generate transgenic zebrafish with reporter genes to facilitate analysis in live fish[37]. Because the zebrafish genome is conserved with humans, information gained from their studies can lead to translational results in humans[9]. The method can be applied to gene expression analysis, such as *in situ* hybridization, to gain additional information regarding the misregulation of genes caused by teratogens.

Ethanol exposure did not significantly affect survival, but it did cause marked defects similar to previous reports[5,23,26]. This demonstrates that our method is reliable in repeating published results. Nitrate had no effect on survival, whereas nitrite did have a significant affect depending on concentration and time of exposure. Longer exposure and higher levels of nitrite had a negative effect on survival, consistent with previous results[5]. It was recently shown that excessive nitrite exposure caused defective heart valve development in zebrafish[27], validating the use of zebrafish to study the mechanism of teratogens.

It is critical to confirm the concentrations of working solutions after they are made. The concentrations of nitrate and nitrite can be measured using modifications of the cadmium reduction (USEPA Method 353.3) and diazotization (USEPA Method 354.1) spectrophotometric methods, respectively[28]. Another critical step is to cover the Petri plate with Parafilm to minimize vitalization of ethanol if this is used as a positive control. If larvae have unexpected mortalities (too high or too low), double-check the calculations and concentrations of the solutions.

Recently, a similar method was used to determine the teratogenic effects of ethanol[29]. Although this method is similar to our method here, it only exposes embryos to ethanol for up to 24 hours, presumably due to the toxicity of exposing embryos to ethanol for a long period of time. In contrast, our method exposes embryos to nitrate and nitrite for several days with replacement of the test solutions daily. This is advantageous for testing less toxic chemicals.

We envision that our method can be applied to test other drugs or specific environmental conditions. However, this method is limited to only testing water-soluble molecules. The light sensitivity of certain chemicals is another factor to consider. If test chemicals are light sensitive, wrap the Petri dishes in aluminum foil to protect from light. Also, the method is not good for testing water taken from a specific environment

because laboratory zebrafish require specific conditions (such as pH and conductivity) for optimal development. Even so, the zebrafish serves as a favorable model to quickly determine developmental defects caused by potential teratogens.

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DISCLOSURES
The authors have nothing to disclose.

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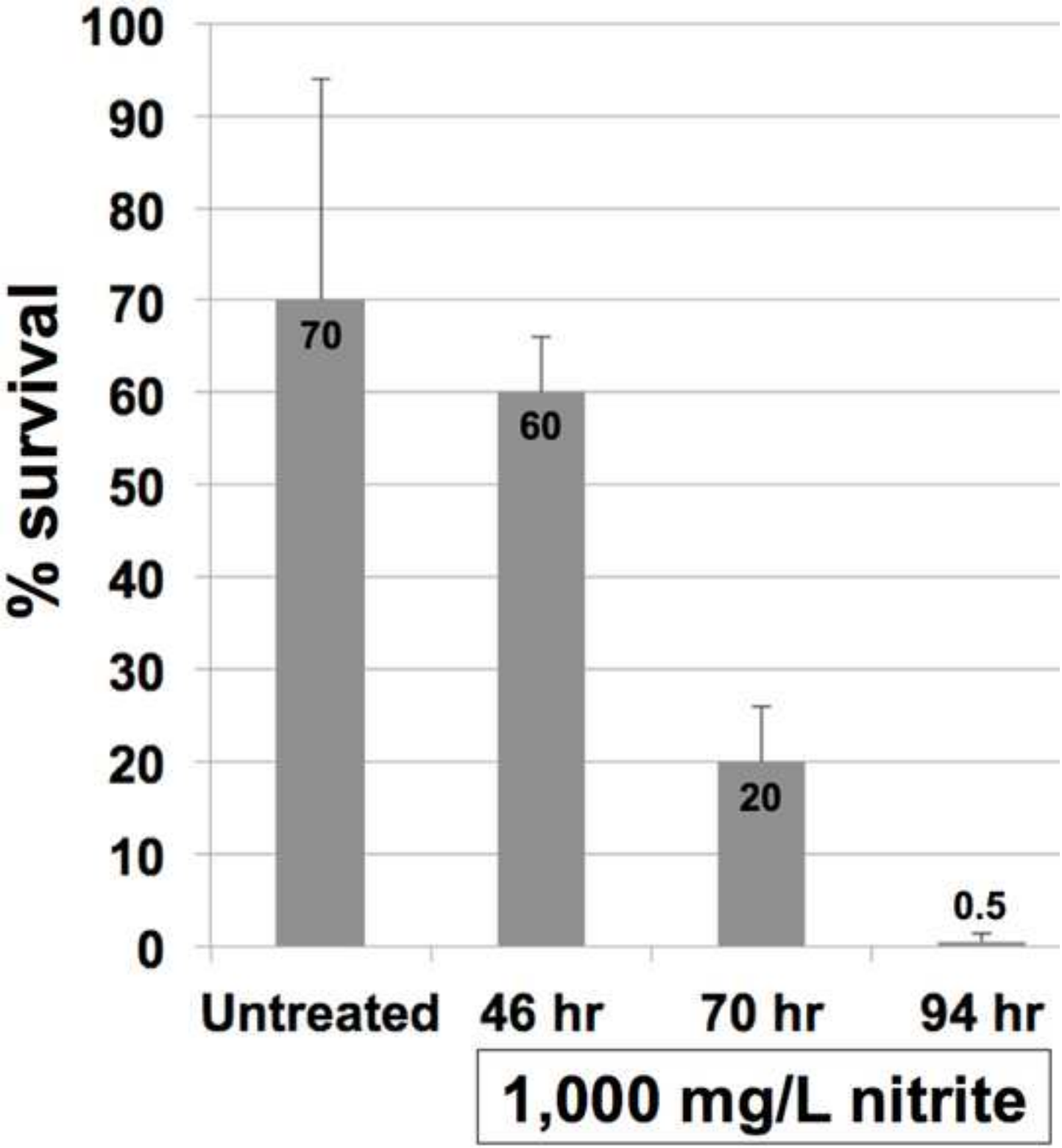

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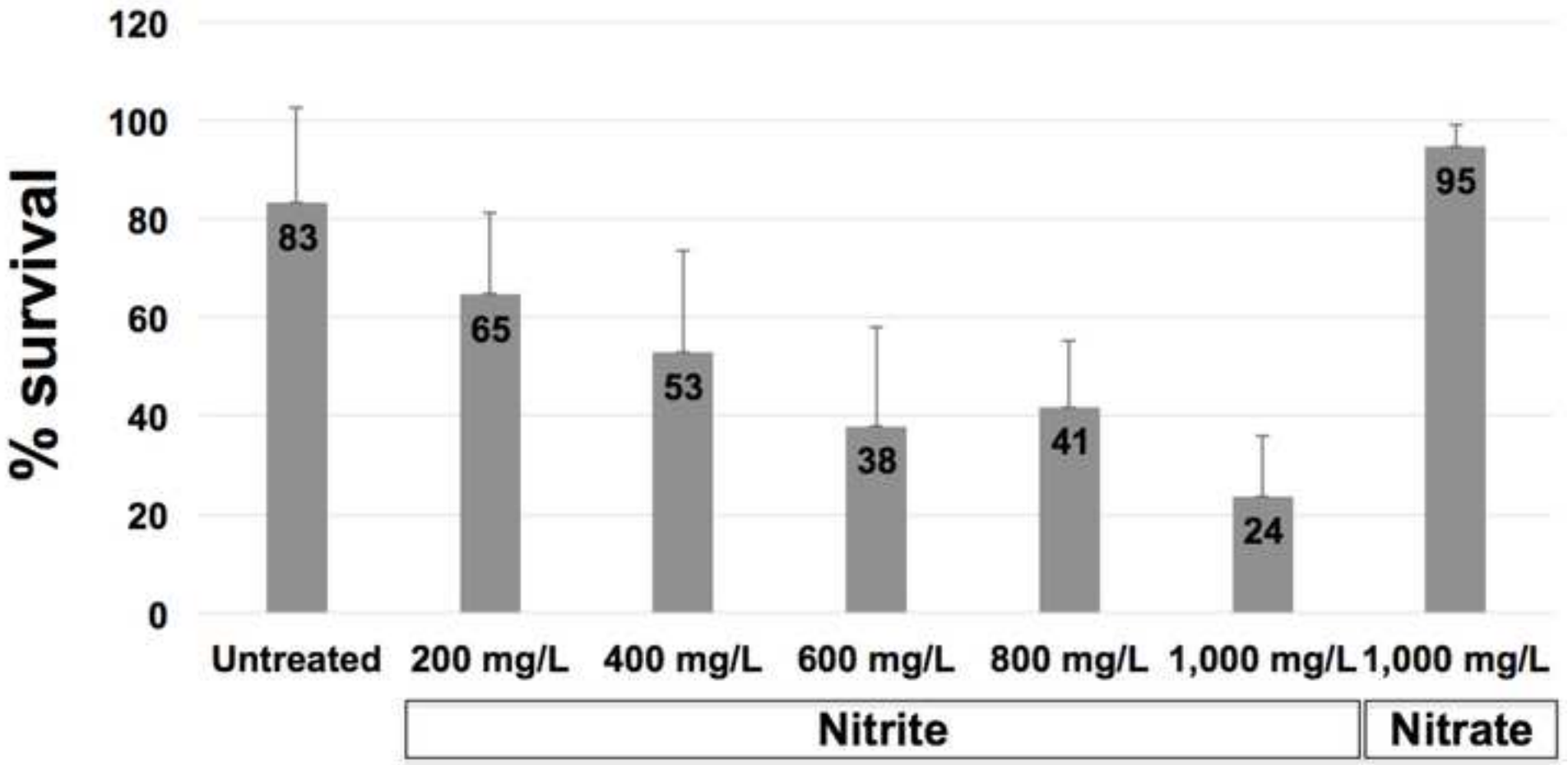

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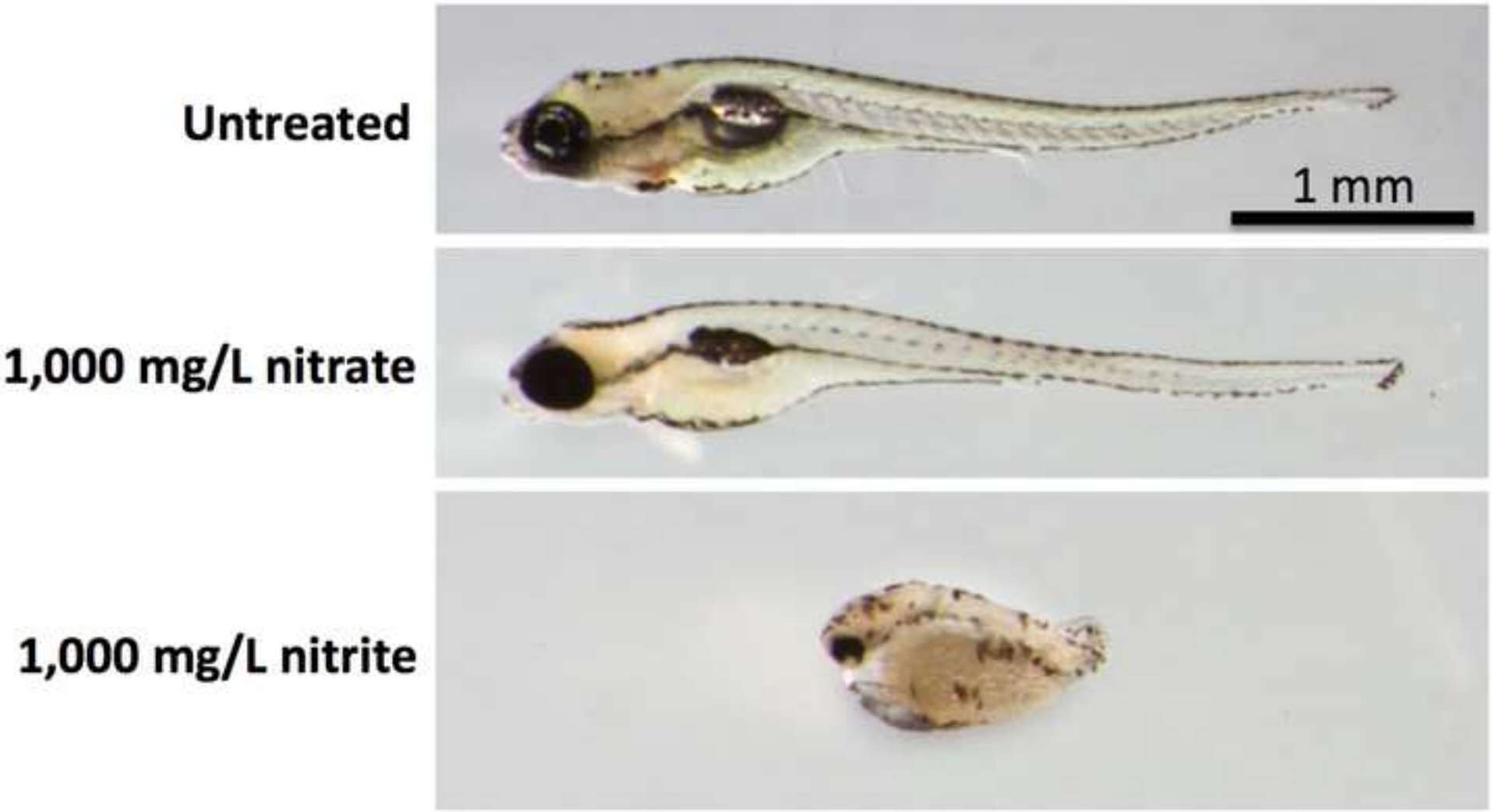

Excel Spreadsheet- Table of Materials/Equipment

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Reagent/Equipment	Company	Catalog Number	Comments/Description
DREL/2010 instrument	Hach	26700-03	
Ethanol	Sigma-Aldrich	E7023	
KIMAX glass Petri Dish	VWR	89001-244	
MS-222	Sigma-Aldrich	E10521	
NitraVer 5 Nitrate Reagent	Hach	14034-46	
NitriVer 3 Nitrite Reagent	Hach	14065-99	
Parafilm	Fisher Scientific	3-374-10	
Paraformaldehyde	Sigma-Aldrich	158127	
S6E stereomicroscope	Leica	10446294	
Sodium nitrate	Fisher Scientific	S343	
Sodium nitrite	Fisher Scientific	S347	
Transfer pipets	Laboratory Products Sales	L320072	
Glass vials	Fisher Scientific	03-338B	

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Manuscript #:

Title of Article: Zebrafish as a model to assess the teratogenic potential of nitrile

Author(s): Keshari, V., Adoeb, B., Simmons, A.E., Simmons, T.W., Diep, C.Q.

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Article Title: Zebrafish as a model to assess the teratogenic potential of nitrite

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Changes made by the Science Editor:
1. There have been edits made to the manuscript.

Changes to be made by the Author(s):
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Reviewer #1:
Manuscript Summary:

In the manuscript "Zebrafish as a model to assess the teratogenic potential of nitrate," authors Keshari, et al., describe the implementation of nitrate testing in zebrafish embryos, which can be used as a system to test other chemicals. Overall, this is a nice manuscript and will be a good resource for scientists looking to adopt zebrafish as a tool for teratogen research.

Major Concerns:

1) Are the nitrate/nitrite solutions light sensitive? How stable are they in solution? What is the justification for the timing of when the solutions are changed?
In terms of anions, nitrite solutions are not sensitive to light in contrast to chlorite which should be stored in the dark. In addition, nitrite solutions are stable up to 6 months at 4 degree C (citation below). To ensure that nitrite was not oxidized to nitrate at 28.5°C during the short duration of our experiments, the solutions were made fresh daily and measured to confirm their concentrations. We have also measured nitrite concentrations after completion of experiments and not detected oxidation to nitrate. Although this procedure for making test solutions was followed as a precaution, it would be recommended for unstable compounds. If compounds were light sensitive, the experiments should be carried out in the dark, assuming zebrafish photoperiod conditions during exposure were not an important factor.

Citation: USEPA (1997) Determination of inorganic anions in drinking water by ion chromatography. Method 300.1. United States Environmental Protection Agency, National Exposure Research Laboratory, Office of Research and Development, Cincinnati, OH.
http://water.epa.gov/scitech/methods/cwa/bioindicators/upload/2007_07_10_methods_method_300_1.pdf

Also, adding a note to the discussion about light sensitivity as a parameter that affects chemical stability, when considering this protocol for use with other compounds, might be useful for many readers.
The following sentences have been added to the discussion to address this concern:

> "The light sensitivity of certain chemicals is another factor to consider. If test chemicals are light sensitive, wrap the Petri dishes in aluminum foil to protect from light."

Minor Concerns:
Minor comments provided below to assist the authors with clarity of the manuscript for readers.
2) Introduction, lines 7073: authors should define what epicanthic folds and clinodactyly are, as these are medical terms not in common scientific usage.
The sentence has been modified to define both terms:

"During human fetal development, common teratogens such as radiation, infectious agents, toxic metals and organic chemicals have been reported to cause defects in epicanthic folds (the skin fold in the upper eye lid) and clinodactyly (curved finger or toe) through morphogenetic errors."

3) Protocol, step 1.4, line 152: How does the researcher know what 1650 embryos looks like? Adding a note with some guidance would be helpful, for example: "Note: Typically, this number of embryos will result from the mating of approximately 15 pairs of zebrafish adults of prime mating ages (39 months of age)."
In step 1.2, we stated that 30 pairs of fish should be set up for mating. We added the following sentence at the end of step 1.2 to address the reviewer's suggestion:

"Typically, about 50% of fish pairs at the prime mating age (6-9 months old) will produce eggs, resulting up to 200 eggs per pair and a maximum of up to 3,000 eggs for this experiment."

4) Protocol, step 2.1, line 163: larvae should not be capitalized. You should also provide a reference to the zebrafish staging series (Kimmel, et al., 1995) for researchers new to the field.
"Larvae" was changed to lower case, and the Kimmel et al., 1995 reference was added.

5) Discussion: A comment about the optical transparency of zebrafish should be added as this is a major benefit of the system, and the use of albino or casper fish can also be mentioned as alternatives to enable greater organ visibility.
The following sentence was modified to include optical transparency:

"Compared to other vertebrates, zebrafish have advantages that include high fecundity, external fertilization, optical transparency, and rapid development."

The following sentence was added to mention the casper fish:

"Available mutants that lack pigmentation (such as the capser zebrafish) also help to enhance visibility of internal organs"

6) Discussion: Linked to the comment above, it would be useful to discuss how transgenesis is possible in zebrafish and that transgenic reporters can be used to gauge tissue or organ development in real time without sacrificing the zebrafish.
The following sentence was added to address this:

"It is also easy to generate transgenic zebrafish with reporter genes to facilitate analysis in live fish."

Additional Comments to Authors:
Overall, nice contribution to JoVE.

Reviewer #2:
Manuscript Summary:

This article explains and outlines a simple, straightforward technique to analyze teratagenic effects of nitrate and nitrite on zebrafish embryos that other scientists will find useful and be able to expand upon with other teratogens of interest. The Title and Abstract are appropriate for the article. With the exception of some minor suggestions of word changes and small additions, the Introduction provides adequate background of the methodological example provided by defining the problem, stating the benefits of using model system with their teratogens of interest, and then finally discussing how these methods are transferable to other teratogenic examples. With the exception of some minor changes, the Protocol is well organized and clear. I feel the suggestions outlined below would make the Protocol even more coherent. Although this is a methods paper, I feel some important changes can be done to the Representative Results to really indicate the power of using

zebrafish as a model system. I do not feel more experimentation needs to take place, but depicting the data as described below may indicate to readers other powerful ways of analysis using these methods.
With the exception of some minor grammatical suggestions, I feel the Discussion is well thought and provides important information for other individuals that wish to use this or similar protocols in their lab.

Major Concerns:
N/A

Minor Concerns:
Long Abstract: Some word choice suggestions: Since you are talking about humans in previous sentences, adding 'other' prior to 'mammalian'(Line 52) may be appropriate. Line 62 perhaps try 'Our results indicate that zebrafish are...'
The following two sentences were modified to address both of these suggestions:

> "However, in other mammalian studies, high nitrite levels do not cause birth defects, although they can lead to poor reproductive outcomes."

> "Our results indicate that the zebrafish is convenient system for studying teratogenic potential of nitrite."

Introduction: Some word choice suggestions: Replace 'like' with 'such as' (Line 76). Add 'in part' (Line 80) so it reads 'This is due, in part, ...' Some phrasing between lines 86-90 seems redundant. Within that same area, also probably move the molecular formulas of nitrate and nitrite from Line 90 to Line 86, when they are fist introduced.
The following two sentences were modified to address these suggestions:

> "Several vertebrate models such as the African clawed frog (Xenopus laevis) and zebrafish (Danio rerio) have been used to determine the molecular pathways affected by teratogens."

> "This is due, in part, to the transparency of the zebrafish embryo, which allows researchers to visualize the developmental defect as it occurs."

The following sentence was deleted to remove redundancy with the previous sentence:

> "Epidemiological studies have shown that nitrate and nitrite, commonly present in farm foods and water, can cause birth defects or spontaneous abortion."

Other suggestions: The authors mention high nitrate levels in water (Line 94). Perhaps it should be explained where the source of these high levels originates from (i.e. is it due to land runoff [external], other organisms within the water sources [internal], or a combination of both).
The following sentence was modified and a reference was added to address this suggestion:

> "Milk from cows fed with high nitrate foods and fish in high nitrate water (mainly from soil runoff) can lead to humans consuming large amounts of nitrate and nitrite."

When explaining the effects of nitrate on humans (beginning at Line 113), it could be more clear as the risk of exposure in infants vs. children vs. adults. If exposed, are all effected in the same way and to the same degree (this question will come up again when looking at your experiment)?
The following sentence was added to clarify the effects in adults:

> "Elevated levels of methemoglobin in adults due to nitrite poisoning results in cyanosis, headache, breathing disorders, and if not treated death due to complications related to vital tissue hypoxia."

Protocol: In Step 1.1, perhaps you should also include the typical light/dark cycle of laboratory zebrafish.

The following sentence was modified to address this suggestion:

> "Maintain zebrafish at 28.5 °C, pH 7, conductivity between 500-1500 µS, and a light/dark cycle of 14 hours light and 10 hours dark."

I was confused by the last sentence in Step 1.4. It seems like you are suggesting to keep unfertilized eggs among the embryos when you perform your experiment. Why? Typically any unfertilized eggs are removed prior to treatments as to not skew survivorship results. Typically I view it as, if something is not fertilized, chances of survival, no matter the treatment, is 0%. So I typically feel removal of unfertilized eggs is important prior to treatment and collection of data.
The following sentence was removed:

> "Mix the embryos by pipetting up and down with a plastic transfer pipet to randomize the unfertilized eggs."

The following sentences were added to address this suggestion:

> "At 1.5 hpf, remove and discard unfertilized eggs with a plastic transfer pipet under a dissecting microscope. Unfertilized eggs are opaque while fertilized eggs are transparent."

In Step 2.1, why did you begin treatments at 2 hpf?
The treatments were begun at 2 hpf to allow for handling time and setup of the experiments (e.g., collection, sorting and transfer of eggs) so that only viable eggs were used and embryos were exposed to the test solutions beginning as closely as possible.

Based on your experience, can effects be seen when treating older embryos? or even adults?
Zebrafish are functionally defined as embryos until hatching from 42 to 70 hours hpf, after which they are referred to as larvae[1]. Other than the acute exposure/lethal effects, and subchronic exposure/sublethal effects of nitrite on larvae (aged 20 to 25 days) and juveniles (aged 2 to 3 months), the effects of nitrite on post-embryonic stages of zebrafish are not well characterized. The 96 hour LC_{50} for larvae exposed to nitrite is 243 mg/L[2], and for juveniles is 243 to 386 mg/L[2,3]. Nitrite concentrations from 73 mg/L cause growth suppression and from 130 mg/L significant growth inhibition of juveniles exposed for 28 days[4].

1. Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B. & Shilling, T. F. Stages of embryonic development of the zebrafish. Developmental Dynamics 203, 253-310 (1995).
2. Voslářová, E, Pištěková, V. & Svobodová, Z. Nitrite toxicity to Danio rerio: effects of fish age and chloride concentrations. Acta Veterinaria Brunensis 75, 107-113, doi.org/10.2754/avb200675010107 (2006).
3. Doleželová, P., Mácová, S., Pištěková, V., Svobodová, Z., Bedáňová, I. & Voslářová, E. Nitrite toxicity assessment in Danio rerio and Poecilia reticulate. Acta Veterinaria Brunensis 80, 309-312. doi:10.2754/avb201180030309 (2011).
4. Voslářová, E, Pištěková, V., Svobodová, Z. & Bedáňová, I. Nitrite toxicity to Danio rerio: effects of subchronic exposure on fish growth. Acta Veterinaria Brunensis 77, 455-460, doi.org/10.2754/avb200877030455 (2008).

In your Introduction, you explained that adults can also be severely effected by nitrate exposure. Perhaps a few sentences clearing this up may be helpful, and if you know, based on experience, if older embryos can also be used, this could open up the protocol to include many more experimental questions.
The following sentence was added to clarify the effects in adults:

> "Elevated levels of methemoglobin in adults due to nitrite poisoning results in cyanosis, headache, breathing disorders[1], and if not treated death due to complications related to vital tissue hypoxia[2,3]."

Although we were interested in the potential teratogenic effects of nitrite exposure during organogenesis (i.e.,

the segmentation and pharygula periods from 10 to 48 hpf) when most deformities occur[4,5], later stages of the zebrafish have been used to study the lethal and sublethal effects of chemicals. For example, juveniles (aged 20 days) have been used to assess the acute toxicity of nitrite[6], larvae (aged 20 to 25 days) and juveniles (aged 2 to 3 months) to investigate the effects of age on acute nitrite toxicity[7], and juveniles exposed for 28 days to evaluate the effects of nitrite on growth[8].

1. Su, Y.-F., Lu, L.-H., Hsu, T.-H., Chang, S.-L. & Lin, R.-T. Successful treatment of methemoglobinemia in an elderly couple with severe cyanosis: two case reports. Journal of Medical Case Reports 6, 290, doi:10.1186/1752-1947-6-290 (2012).
2. Harvey, M., Cave, G. & Chanwai, G. Fatal methaemoglobinaemia induced by self-poisoning with sodium nitrite. Emergency Medicine Australasia 22, 463–465, doi:10.1111/j.1742-6723.2010.01335.x (2010).
3. Nishiguchi, M., Nushida, H., Okudaira, N. & Nishio, H. An autopsy case of fatal methemoglobinemia due to ingestion of sodium. Forensic Research 6, 1000262, doi:10.4172/2157-7145.1000262 (2015).
4. Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B. & Shilling, T. F. Stages of embryonic development of the zebrafish. Developmental Dynamics 203, 253-310 (1995).
5. McCollum, C. W., Ducharme, N. A., Bondesson, M. & Gustafsson, J.-A. Developmental toxicity screening in zebrafish. Birth Defects Research (Part C) 93, 67-114, doi: 10.1002/bdrc.20210 (2011).
6. Doleželová, P., Mácová, S., Pištěková, V., Svobodová, Z., Bedáňová, I. & Voslářová, E. Nitrite toxicity assessment in Danio rerio and Poecilia reticulate. Acta Veterinaria Brunensis 80, 309-312. doi:10.2754/avb201180030309 (2011).
7. Voslářová, E, Pištěková, V. & Svobodová, Z. Nitrite toxicity to Danio rerio: effects of fish age and chloride concentrations. Acta Veterinaria Brunensis 75, 107-113, doi.org/10.2754/avb200675010107 (2006).
8. Voslářová, E, Pištěková, V., Svobodová, Z. & Bedáňová, I. Nitrite toxicity to Danio rerio: effects of subchronic exposure on fish growth. Acta Veterinaria Brunensis 77, 455-460, doi.org/10.2754/avb200877030455 (2008).

Minor note, in Line 163, larvae should not be capitalized.
This has been fixed.

Representative Results: Data collection was straightforward and described well, however the way the data is presented could be easily improved. Although this journal focuses on methodology, I feel presenting the data in a different was could show the reader the power of zebrafish as a model system in this study. I'm referring specifically to Figures 2 and 3. Instead of simply showing the percentage survival at the end of each treatment, why not instead show survivorship curves? In the protocol, it is stated embryos are checked daily and dead embryos are removed. Including survivorship curves showing survivorship every 24 hpf (in a line graph or scatter plot form) is a much more powerful way to represent the data. This way, trends can be seen as to whether embryos are dramatically effected early, have a steady decline over the time of the study, or if they are effected more during the later period of the study.
We thank the review for this suggestion. Unfortunately, my students did not record how many dead embryos they removed each day. Therefore, we currently lack the data to generate a daily survival curve.

In Figure 4, it strongly appears that the untreated and 1,000 mg/L treated embryos are at different developmental timepoints. Developmental delay was stated as an occurrence with these treatments. Perhaps this should be mentioned to the reader again in the figure legend, or alternatively, developmentally match your embryos based on features instead of hours to get embryos that are at the same developmental timepoints.
The middle panel of Figure 4 has been replaced with a developmentally matched embryo (compared to the untreated embryo on the top panel). The figure legend has also been modified to reflect this change.

Discussion:
Minor suggestions Change
'The' to 'This' (Line 280).

The following sentence was modified to address this suggestion:

> "This method can be applied to gene expression analysis, such as in situ hybridization, to gain additional information regarding the misregulation of genes caused by teratogens."

Add 'of' between 'concentrations' and 'nitrate' (Line 294).
Change 'the' to 'this' (Line 310).
The following sentence was modified to address this suggestion:

> "The concentrations of nitrate and nitrite can be measured using modifications of the cadmium reduction (USEPA Method 353.3) and diazotization (USEPA Method 354.1) spectrophotometric methods, respectively."

In Figures 2, 3, and 4, the abbreviation of liters should be a capitalized 'L'.
The changes have been made according to the suggestion.

Reviewer #3:
Manuscript Summary:
The manuscript describes a protocol for assessing the teratogenic potential of nitrite using zebrafish as a model.

Major Concerns:
Instead of mixing the embryos by pipetting up and down with a plastic transfer pipette to randomize the unfertilized eggs (Line 154155), it is strongly suggested to remove the unfertilized eggs or the embryos with obviously abnormal development from the pool embryos by observing the embryos at 2 hpf under dissecting microscope. The toxic effect of a given teratogen on the zebrafish embryos would be impossible to be evaluated if the embryos in the control group exhibits very low survival rate (as shown in untreated in Figure 2). For example, the conclusion made in Line 240-Line 241 "exposure to 1,000 mg/L for 46 hours decreased survival by 10% (compared to the untreated embryos) (Figure 2)" would not be reached if the data were examined by a statistics test (eg. Student t-test).
The following sentence was removed to address the suggestion of removing unfertilized eggs:

> "Mix the embryos by pipetting up and down with a plastic transfer pipet to randomize the unfertilized eggs."

The following sentences were added to address the suggestion of removing unfertilized eggs:

> "At 1.5 hpf, remove and discard unfertilized eggs with a plastic transfer pipet under a dissecting microscope. Unfertilized eggs are opaque while fertilized eggs are transparent."

The following paragraph was also modified to more accurately describe the data in Figure 2:

> "Treatment with nitrite resulted in mild to severe effects on survival, depending on the time of exposure. For example, exposure to 1,000 mg/L for 94 hours severely affected survival compared to shorter exposure times (Figure 2)."

Minor Concerns:
N/A

Reviewer #4:
Manuscript Summary:

This manuscript describes the method used to determine the validity of zebrafish as a model system for assessing the developmental effects of various molecules. More specifically, the authors study the effects of nitrite, by increasing exposure time and concentration, on early zebrafish development. They find that increasing both results in a decrease in survival. They also show that treatment with nitrite results in developmental defects similar to those seen with ethanol, their positive control. Interestingly, treatment with 1000 mg/L nitrate does not affect survival. The manuscript is well written and delineates a very useful methodology for quickly assessing the effects of treatment with various molecules/compounds.

Major Concerns:
N/A

Minor Concerns:
Protocol:
1)Step 1.4: The authors mention that embryos are pipetted up and down to randomize the unfertilized eggs. Are these unfertilized eggs eventually removed? If not, they could increase the amount of death (and variability) following treatment. Since treatment is performed at 2hpf, it would be easy to ensure that all treated embryos have been fertilized (and to remove the unfertilized ones).
The following sentence was removed:

> "Mix the embryos by pipetting up and down with a plastic transfer pipet to randomize the unfertilized eggs."

The following sentences were added to address this suggestion:

> "At 1.5 hpf, remove and discard unfertilized eggs with a plastic transfer pipet under a dissecting microscope. Unfertilized eggs are opaque while fertilized eggs are transparent."

2)For all of the treatments, the authors should include the solvent or diluent used.
The following sentence was modified to address this for ethanol:

> "2.2) For 3 Petri dishes (each containing 50 embryos) at 2 hpf, remove the E3 buffer with a transfer pipet and add 50 mL of 300 mM ethanol diluted in E3 buffer."

The following sentence was modified to address this for sodium nitrite:

> "2.4) For 9 Petri dishes (each containing 50 embryos) at 2 hpf, remove the E3 buffer with a transfer pipet and add 50 mL of 1,000 mg/L sodium nitrite dissolved in E3 buffer."

The following sentence was modified to address this for sodium nitrate:

> "2.6) For 3 Petri dishes (each containing 50 embryos) at 2 hpf, remove the E3 buffer with a transfer pipet and add 50 mL of 1,000 mg/L sodium nitrate dissolved in E3 buffer."

3)In the discussion, the authors mention a method used to confirm the working concentrations of nitrate and nitrite but this is not included in the methodology. While the inclusion of the specific steps of this method might be outside of the scope of this manuscript, the authors should mention the need to confirm concentrations in the method section.
The following sentence was added to step 2.4 to address this suggestion:

> "Confirm the concentration of the stock nitrite solution beforehand using a modification of the diazotization (USEPA Method 354.1) spectrophotometric method."

The following sentence was added to step 2.6 to address this suggestion:

> "Confirm the concentration of the stock nitrate solution beforehand using a modification of the cadmium reduction (USEPA Method 353.3) spectrophotometric method."

Results:
1)Figures 1 and 4: Scale bars should be included with the images.
The scale bars have been added to Figure 1 and 4.

2)Figure 1: The authors should also highlight the yolk sac edema.
The yolk sac edema has been highlighted with an arrowhead and the figure legend was modified as follows:

> "Embryos treated with ethanol showed pericardial edema (arrow), swim bladder noninflation (dashed line), yolk sac edema (arrowhead), and craniofacial defects (data not shown).

3)Figures 24:
The authors should capitalize "L" in mg/L to complement what is written in the figure legends.
This has been changed according to the suggestion.

4)Figure 4: Since the authors mention/describe the phenotypes following treatment with 1000 mg/L nitrite, the third panel of the figure should reflect this treatment concentration. If this is not possible, they should include the percent of larvae at 200 mg/L nitrite that display the severe phenotype shown (provided that there is a range of phenotypes).
The third panel of Figure 4 has been changed to use an embryo that was treated with 1000 mg/L nitrite.

Additional Comments to Authors:
Grammatical Corrections:
Abstract
1)Line 60: Sentence should read "Increasing the concentration of nitrite also adversely affects survival…"
The following sentence was modified to reflect this suggestion:

> "Increasing the concentration of nitrite also adversely affects survival, whereas nitrate does not."

2)Line 63: "…convenient system for studying the teratogenic potential of nitrite."
The following sentence was modified to reflect this suggestion:

> "Our results indicate that the zebrafish is a convenient system for studying the teratogenic potential of nitrite."

Introduction
1)Line 68: "abnormality" should be plural
The following sentence was modified to reflect this suggestion:

> "Teratogenesis is a process that disrupts the normal development of an embryo or fetus by causing permanent structural and functional abnormalities, growth retardation, or miscarriage in severe cases."

2)Line 71: there should be a comma after toxic metals
The following sentence was modified to reflect this suggestion:

> "During human fetal development, common teratogens such as radiation, infectious agents, toxic metals,

and organic chemicals have been reported to cause defects in epicanthic folds (the skin fold in the upper eye lid) and clinodactyly (curved finger or toe) through morphogenetic errors."

3)Line 88-90:
Sentence beginning with "Epidemiological studies…" should be removed. It is redundant with the previous statement.
This sentence has been deleted.

4)Line 115: Sentence should read "Chronic exposure to a high level of nitrate…"
The following sentence was modified to reflect this suggestion:

"Chronic exposure to a high level of nitrate is associated with urination and spleen hemorrhaging."

5)Line 119: Sentence should read "Prenatal exposure to nitrate at high levels…neural tube and musculoskeletal defects.
The following sentence was modified to reflect this suggestion:

"Prenatal exposure to nitrate at high levels has been associated with neural tube and musculoskeletal defect."

6)Line 126: Sentence should read "…different concentrations and different lengths of time."
The following sentence was modified to reflect this suggestion:

"Embryos were exposed to nitrite at different concentrations and different lengths of time."

Protocol
1)Step 2.9: Sentence should read "Orient the larvae so that the anterior is to the left…"
The following sentence was modified to reflect this suggestion:

"Orient the larvae so that the anterior to the left and the dorsal is to the top of the field."

2)Multiple steps: larvae should not be capitalized (embryos/larvae)
All of the "embryo/larvae" phrases have been changed according to the suggestion.

Discussion
1)Line 279: Sentence should read "Because the zebrafish genome is conserved with humans…"
The following sentence was modified to reflect this suggestion:

"Because the zebrafish genome is conserved with humans, information gained from their studies can lead to translational results in humans."

2)Line 285: Sentence should read "…cause marked defects similar to previous reports."
The following sentence was modified to reflect this suggestion:

"Ethanol exposure did not significantly affect survival, but it did cause marked defects similar to previous reports."

3)Line 294: Sentence should read "The concentrations of nitrate and nitrite…"
The following sentence was modified to reflect this suggestion:

"The concentrations of nitrate and nitrite can be measured using modifications of the cadmium reduction (USEPA Method 353.3) and diazotization (USEPA Method 354.1) spectrophotometric methods, respectively."

4)Line 308: Remove comma after drugs
The following sentence was modified to reflect this suggestion:

"We envision that our method can be applied to test other drugs or specific environmental conditions."